MICROBIOTEST PROTOCOL

EFFICACY TESTING OF ANTIBACTERIAL DISHWASHING DETERGENT, Ultra Density and Regular Density

Staphylococcus aureus and *Salmonella enterica*

Testing Facility
Microbiotest
105 Carpenter Drive, Suite B
Sterling, VA 20164

Prepared for
COLGATE-PALMOLIVE COMPANY
909 River Road
Piscataway, NJ 08855

December 10, 2007

OBJECTIVE:

This test is designed to support antibacterial claims ("kills 99.9% of bacteria") on soiled dishware. The method determines the efficacy of products intended to be used for one-step cleaning and antibacterial on surface of soiled dishware and is based on the Germicidal Spray Products Test, *Official Methods of Analysis*, Sixteenth edition, 1995, AOAC. Testing will be conducted to support additional efficacy claims consistent with Pesticide Assessment Guidelines, Subdivision G: Product Performance.

TESTING CONDITIONS:

Two substances will be evaluated using a total of ten replicates per microorganism, per lot of test substance will be evaluated using three lots of each test substance, one of which is at least sixty days old for each test substance. A non-porous, glass slide carrier will be inoculated with *Staphylococcus aureus* and *Salmonella enterica*, and treated under exposure conditions (see last page) consistent with the product's recommended use instructions. Following exposure, carriers and excess product run-off will be transferred to neutralizing broth and all liquids serially diluted and cultured.

MATERIALS:

A. Test Substances - Supplied by the sponsor: (see last page).

Test Substance 1: Ultra Density
Test Substance 2: Regular Density

Reserve samples of each batch of test substance is being taken by Tom Connors of Colgate-Palmolive, therefore, MICROBIOTEST will not take additional samples.

The sponsor assures MICROBIOTEST testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

MICROBIOTEST will retain all unused test substances for a period of at least three months after completion of the study, and will then be returned to the sponsor or discarded in a manner that meets the approval of the safety officer of the laboratory following sponsor approval.

B. Materials supplied by MICROBIOTEST including but not limited to:

1. Test Systems - Challenge organisms

   a. *Staphylococcus aureus*, ATCC 6538 (SA)
   b. *Salmonella enterica*, ATCC 10708 (SE)
   c. Age of test system - 48-54 hours
   d. Source of supply - ATCC
   e. Test System Identification – The microorganisms will be placed in a tube and the tube will be labeled with the Genus/species and ATCC number. The test tubes will be in a rack labeled with the project number, the identification of the test substance (including the lot number), the test system and the test replicates.
   f. Justification for Selection of Test System – SA and SE were selected since the organisms are the required organisms to be used by the EPA.

2. Media and reagents:

   a. Nutrient Broth (NB).
   b. DE Neutralizing Broth (DE).
   c. Sterile saline (SS).
   d. Trypticase Soya Agar (TSA).
   e. Heat Inactivated Horse Serum.
   f. Phosphate Buffered Saline (PBS).
   g. PBS containing 0.5% polysorbate 80 (PBS+).

3. Miscellaneous laboratory equipment and supplies.

4. Test surface: Glass Slide Carriers.

EXPERIMENTAL DESIGN:

A. Dose Solution Preparation

The test substance will be prepared in accordance with the instructions stipulated on the last page of the protocol.

B. Test Substance Dosage

1. Dosage Levels

See Miscellaneous Information page for each test substance

2. Route and Frequency of Administration, Duration, and Reason for Choice

Each test substance will be spread one time by applying the substance to a glass slide and a sterile glass rod used to spread the material back and forth across the slide for approximately 15 seconds. Exposure duration will be 30 seconds

The dosing procedure is the preferred technique based on the specific method.

C. Dose Solution Analysis

Test Site: Charles River Laboratories
640 N. Elizabeth Street
Spencerville, OH 45877

Principal Investigator: M. Gardner Clemons
Senior Manager, Laboratory Sciences
Dose Formulation Analysis
ph 419-647-4196 x 350
f 419-647-6560
gardner.clemons@crl.com

Samples of the dose solution will be taken by Microbiotest and shipped to Charles River Laboratory for concentration analysis. Dose solutions will be analyzed by HPLC according to Colgate Palmolive method LAB 1110 "Lactic Acid Products, Raw Materials, and Dilute Aqueous Solutions by HPLC".

D. Inocula preparation:

Bacteria from stock cultures will be transferred into NB and incubated at 37±2C. Daily transfers will be made for at least three (but less than 10) consecutive days. Tubes of NB will be inoculated using one loopful of inoculum for each tube. After a 48-54 hour incubation period, the cultures will be pooled into a sterile flask.

In order to concentrate each inoculum, the cultures will be centrifuged for five minutes at 4000 rpm. The supernatant will be discarded and the pellet will be re-suspended using NB to 1/10 of the original volume, or as necessary to achieve the desired starting concentration.

Organic load (5% heat Inactivated horse serum) will be added to the cultures.

E. Carrier preparation:

The carriers will be sterilized by placing them in evaporating dishes matted with filter paper, heating them in a hot air oven for two hours at 180C, cooling and storing them at room temperature until use.

F. Carrier inoculation:

Each prepared culture will be mixed 3-4 sec on a Vortex-type mixer, then allowed to settle for ten min before being used. A 0.01-0.03 mL aliquot of each inoculum will be transferred onto a one-sq. inch area on the sterile carriers (in sterile Petri dishes) and immediately spread uniformly over the entire area with a sterile glass rod. Each dish will be covered promptly and the operation will be repeated for the rest of the carriers. Carriers will be dried for 20-40 min at 37±2C.

G. Test:

Ten carriers for each organism per lot of test substance will be treated with three mL of the prepared test substance (see last page). The test material will be spread on the carrier for a total of 30 seconds and added to neutralizer at the end of the total exposure time as specified by the sponsor. (Spreading will be defined as: once the test substance has been applied to a glass slide, a sterile glass rod will be used to spread the material back and forth across the slide for approximately 15 seconds. The spreading will continue in an up and down

motion for the remainder of the 30 seconds.) At the completion of spreading, the excess product run-off will be collected; and then the carrier will be transferred to a tube containing 20 mL DE. For the run-off sample, one-mL will be collected and transferred to a tube containing 10 mL of DE.

Tubes containing carriers will be subjected to ultra sound for five minutes. Both the contents of the sonicated carrier tubes and the neutralized run-off sample will be serially diluted in tenfold increments (if necessary) and duplicate one-mL aliquots from selected dilutions will be plated in duplicate TSA pour plates.

Once solid, all plates will be inverted and incubated for two days at 37±2C in ambient air.

H. Controls:

1. Numbers controls:

For each challenge organism, three carriers will be inoculated and dried as for the test. Three mL of PBS+ will be dispensed over each inoculated carriers, a glass rod will be used to spread the PBS+ over the surface of the carrier for 30 seconds in the same manner as the test. One-mL of the run-off will be collected and serially diluted tenfold in PBS dilution blanks. The carriers will be placed in tubes containing 20 mL PBS+, the tubes subjected to ultra sound for five minutes and the solutions will be serially diluted tenfold in PBS dilution blanks. For both the run-off and carrier solutions, duplicate one-mL aliquots of selected dilutions will be plated in TSA pour plates and incubated with the test.

2. Neutralizer effectiveness control:

For each challenge microorganism, a single sterile carrier will be exposed to the prepared test substance for the required contact time in the same manner as the test. The carrier will be transferred to a tube containing 20 mL of DE. For the run-off sample, one-mL will be collected and transferred to a tube containing 10 mL of DE. Fewer than 10 CFU/mL of the appropriate challenge organism will be added to each tube and the count of the bacteria inoculated into these tubes will be confirmed in duplicate using TSA. The tubes and plates will be incubated with the test.

3. Sterility control:

   Sterile carriers will be added to two tubes of DE and the tubes will be incubated with the test.

4. Organism confirmation:

   For each challenge organism, a randomly selected colony from the Numbers control plates, and if applicable, a randomly selected colony from a test plate will be confirmed by colony morphology and Gram stain according to extant SOPs.

PRODUCT EVALUATION CRITERIA:

The test material meets minimum effectiveness requirements for claiming "kills 99.9% of bacteria on dishware" if there is a three log (99.9%) reduction, relative to the numbers controls, for both carriers and the excess product run-off for each lot and microorganism evaluated. The test results will be calculated for each test replicate.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- The carrier numbers controls should be at least 1.0×10^6 CFU/carrier but does not exceed 1.0×10^7 CFU/carrier.
- The excess product run-off numbers controls should be at least 1.0×10^4 CFU/mL but does not exceed 1.0×10^7 CFU/mL.

DATA PRESENTATION:

The final report will include the following information in tabular form:

- The results for the test carrier and product run-off counts.
- The results for all controls.
- The log reduction results for the test carrier and product run-off.

PROPOSED STATISTICS

Means will be calculated for each organism carrier and run-off control.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned prior to initiation of the test. Resumes for the technical personnel are maintained and are available on request. This study will be conducted in the Applied Microbiology Laboratory at MICROBIOTEST, 105 Carpenter Drive, Sterling, Virginia 20164.

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test substance records, final report, and correspondence between MICROBIOTEST and the sponsor will be stored in the archives at MICROBIOTEST, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site. All above items will be maintained by MICROBIOTEST indefinitely, unless requested by the sponsor to be transferred to another archive facility.

Analytical data supporting the dose analysis will be maintained by Charles River Laboratories, 640 N. Elizabeth Street, Spencerville, OH 45877.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test substance; challenge microorganism used; media and reagent identification; and the type of neutralizers employed in the test will be addressed in a project sheet issued separately. The date the study director signs project sheet number one will be the initiation date. All project sheets will be forwarded to the study sponsor.

PROTOCOL AMENDMENTS

The study director may verbally authorize protocol changes; however, a written amendment must be issued by the study director within two weeks, and must be signed and dated by the study director and sponsor/management. Any amendments will include a justification statement for the protocol change.

PROTOCOL AND SOP DEVIATIONS

Deviations from the Protocol and Standard Operating Procedures will be promptly reported to the Study Director. Major deviations will be reported to the Study Director immediately. The Study Director will issue a written protocol or SOP deviation within two weeks of discovery. The deviation will include a study impact statement; i.e., whether the deviation affected the quality or integrity of the study.

QUALITY ASSURANCE

Quality Assurance in accordance with the applicable Standard Operating Procedures will audit the protocol, critical phases, raw data, and the final report. All QAU audit reports will be issued to the study director and study director management. The sponsor will be provided copies of QA audit reports if requested.

REPORT FORMAT:

MICROBIOTEST employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification
- Test substance identification
- Type of assay and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria
- Signed Quality Assurance and Compliance Statements (if applicable)

The Sponsor will be provided copies of a QA audited draft report for review and

comments. Final report will be issued after written agreement of Sponsor Representative.

The Sponsor will be provided with three exact copies of the final report.

MISCELLANEOUS INFORMATION:

This information must be completed by the sponsor before initiation of the study:

A. Name & address COLGATE-PALMOLIVE COMPANY
909 River Road
Piscataway, NJ 08855

B. Test Substance 1: Ultra Density

Lot No. 1: UCB4FU2LP-13 Lot No. 2: UCB5FU2LP-13
Lot No. 3: UCB6FU2LP-13 (at least 60 days aged)

Active ingredient(s): 2% Lactic Acid

Contact time: 30 seconds (inclusive of a 30 second spread time)

Contact temperature: ambient room temperature

Volume of test material applied to each carrier: 3 mL

Use Dilution to be tested: 1:20

Diluent: Sterile deionized water

C. Organic load – serum added to achieve 5% in the inoculum: ■ yes ☐ no.

D. Precautions/storage conditions: refer to MSDS or certificate of analysis
■ provided ☐ not provided

E. Additional information: Not applicable

REPORT HANDLING:

The sponsor intends to submit this information to (CHOOSE ONE ONLY):
■ EPA ☐ FDA ☐ Health Canada ☐ CAL DPR
☐ ARTG ☐ non GLP ☐ other _____

PROTOCOL APPROVAL:

Sponsor: Catherine B Lewus Date: 12/10/07

Printed Name: Catherine B. Lewus

MISCELLANEOUS INFORMATION:

This information must be completed by the sponsor before initiation of the study:

A. Name & address COLGATE-PALMOLIVE COMPANY
909 River Road
Piscataway, NJ 08855

B. Test Substance 2: Regular Density

Lot No. 1: UCB4FR2LP-16 Lot No. 2: UCB5FR2LP-16

Lot No. 3: UCB6FR2LP-16 (at least 60 days aged)

Active ingredient(s): 2 % Lactic Acid

Contact time: 30 seconds (inclusive of a 30 second spread time)

Contact temperature: ambient room temperature

Volume of test material applied to each carrier: 3 mL

Use Dilution to be tested: 1:20

Diluent: Sterile deionized water

C. Organic load – serum added to achieve 5% in the inoculum: ■ yes ☐ no.

D. Precautions/storage conditions: refer to MSDS or certificate of analysis
■ provided ☐ not provided

E. Additional information: Not applicable

REPORT HANDLING:

The sponsor intends to submit this information to (CHOOSE ONE ONLY):
■ EPA ☐ FDA ☐ Health Canada ☐ CAL DPR
☐ ARTG ☐ non GLP ☐ other _____

PROTOCOL APPROVAL:

Sponsor: Catherine B. Lewus Date: 12/10/07

Printed Name: Catherine B. Lewus